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published in
Environmental Toxicology and Pharmacology
2020

DOI (link to publisher)
10.1016/j.etap.2020.103460

document version
Publisher's PDF, also known as Version of record

Environmental Toxicology and Pharmacology 80 (2020) 103460

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

High resolution effect-directed analysis of steroid hormone (ant)agonists in surface and wastewater quality monitoring

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ARTICLE INFO

Keywords:
Endocrine disruption
Anti-androgen
Bioassay
Non-target screening
High throughput
Bioanalytical tool

ABSTRACT

Monitoring of chemical water quality is extremely challenging due to the large variety of compounds and the presence of biologically active compounds with unknown chemical identity. Previously, we developed a high resolution Effect-Directed Analysis (EDA) platform that combines liquid chromatography with high resolution mass spectrometry and parallel bioassay detection. In this study, the platform is combined with CALUX bioassays for (anti)androgenic, estrogenic and glucocorticoid activities, and the performance of the platform is evaluated. It appeared to render very repeatable results, with high recoveries of spiked compounds and high consistency between the mass spectrometric and bioassay results. Application of the platform to wastewater treatment plant effluent and surface water samples led to the identification of several compounds contributing to the measured activities. Eventually, a workflow is proposed for the application of the platform in a routine monitoring context. The workflow divides the platform into four phases, of which one to all can be performed depending on the research question and the results obtained. This allows one to make a balance between the effort put into the platform and the certainty and depth by which active compounds will be identified. The EDA platform is a valuable tool to identify unknown bioactive compounds, both in an academic setting as in the context of legislative, governmental or routine monitoring.

1. Introduction

Our modern society uses thousands of different organic chemical compounds that end up in the water cycle (Houtman, 2010; Stefanakis AIB, 2015) via discharge of sewage, agricultural and industrial waste and often incomplete removal in wastewater treatment (Petrovic et al., 2006, 2003). This is worrying, as these compounds might adversely affect public health via drinking water (DW) prepared from surface water and the health of ecosystems. Proper monitoring of chemical water quality is extremely challenging due to the large variety of compounds that should be analysed and due to the presence of many biologically active compounds with unknown chemical identity (Houtman, 2010; van Wezel et al., 2010; Weiss et al., 2011).

Mechanism-based in vitro bioassays – biological test systems that directly estimate a specific biological effect in a sample - are used more and more to investigate compounds with biological, possibly toxic activities (Schriks MB et al., 2015). Bioassays are important tools for water boards to monitor chemical water quality in their catchments and for DW companies to survey the chemical water quality in their sources for the preparation of DW (Escher et al., 2014; Houtman et al., 2018; Luigi, 2011; van der Oost et al., 2017a).

Nowadays, the panel of effects for which bioassays have been developed has grown considerably. As biological pathways that are identified for humans and ecology are numerous, certain molecular key events of adverse outcome pathways of effects (Ankley et al., 2010) have been pinpointed that should ideally be assessed by bioassays to cover the most relevant health endpoints in water samples, for both human health (Schriks MB et al., 2015) and aquatic ecology (Escher et al., 2018; Neale et al., 2017; van der Oost et al., 2017b). These include xenobiotic metabolism, reactive modes of action, cytotoxicity, developmental toxicity and endocrine disruption. If bioassays are applied for water quality assessment it should be decided which level of response is considered to indicate a human or environmental risk. To this purpose suites of effect-based 'trigger values' (EBT) have been derived in recent years (Escher et al., 2018; van der Oost et al., 2017b; Brand et al., 2013). These trigger values differentiate between low risks

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https://doi.org/10.1016/j.etap.2020.103460
Received 17 February 2020; Received in revised form 15 July 2020; Accepted 27 July 2020
Available online 29 July 2020
1382-6689/

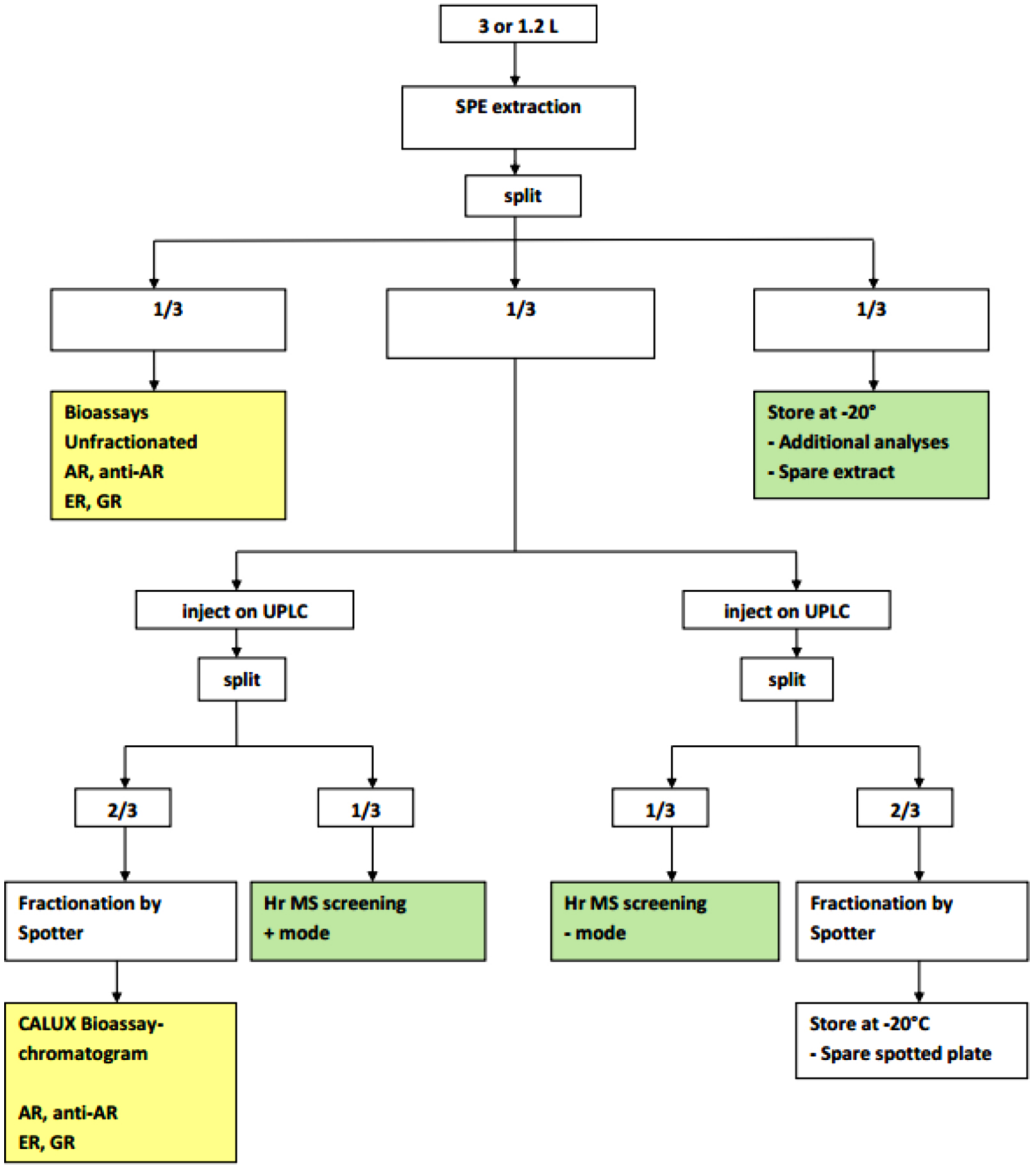

Fig. 1. Flow diagram of the experimental approach of the high resolution Effect-Directed Analysis (EDA) platform for water samples. Colourless blocks refer to sample treatment steps, yellow blocks represent bioassay measurements, green blocks represent chemical analyses. A schematic representation of the platform with an explanation for each step is provided in the Supplemental Information Figure S1.

for adverse effects if bioassay responses are below EBT, and increased risks for adverse effects on human or ecosystem health if bioassay responses exceed the EBT.

Endocrine disruption is an important endpoint as the presence of endocrine disrupting compounds –able to mimic or antagonize steroid hormonal action in the body – in surface waters may cause adverse effects on aquatic organisms (Sumpter and Jobling, 2013; Jobling et al., 1998). There is also concern for human health as there are indications that exposure to endocrine disrupting compounds (EDCs) might play a role in the aetiology of health problems like reduced fertility, obesitas and type II diabetes mellitus (Kabir et al., 2015; Touraud et al., 2011; Heindel et al., 2015; Ehrlich et al., 2016). EDCs include natural hormones, human and veterinary pharmaceuticals, metabolites thereof and many industrial compounds able to interfere with hormonal action, such as hormone receptor activation (Sumpter, 2005).

Although estrogens and androgens in the environment have received most research attention, awareness is growing for other hormonal (and corresponding antagonistic) activities, as also compounds with e.g. glucocorticoids, and progestogenic activities are being used by humans and livestock and consequently released into the environment.

The presence of EDC in water can be determined chemically or biologically by different reporter gene bioassays (Escher et al., 2014; Macova et al., 2011; Neale et al., 2012; Van der Linden et al., 2008). Once activity is found in the bioassay, the identity of the causative compounds is often required to enable risk assessment and source tracking of the EDC. However, as bioassays are usually performed using extracts of environmental samples, they assess the total activity of the whole mixture without separating compounds contributing to the activity and determining their chemical identity. This is a principal difference from chemical analyses that use chromatographic separation of compounds before detecting them one by one.

Effect-directed analysis (EDA) is a powerful tool to identify the drivers of bioassay activity in complex environmental samples. It combines bioassay analysis with chromatographic separation (fractionation) and chemical analysis of active fractions (Brack et al., 2016; Houtman et al., 2011). In the past, the successful application of EDA was often hampered by limited chromatographic resolution and identifying power (Houtman et al., 2011). To overcome this, a high

resolution EDA platform was developed applying high resolution fractionation and LC–MS identification (Jonker et al., 2015). The platform allows construction of bioassay chromatograms that can directly be correlated to MS chromatograms recorded in parallel, allowing straightforward pinpointing of accurate masses of active compounds. The applicability of the novel platform was demonstrated in studies published by Zwart et al. (2020), 2018 and Jonker et al. (2016).

The present study has three main objectives:

1 To evaluate the method performance of the HT-EDA-platform. The platform appeared to be powerful in the aforementioned demonstration studies (Jonker et al., 2016, 2019; Kabir et al., 2015). However, additional evidence of the robustness of the platform is needed before it can be applied in a more structural, legislative or governmental context. Therefore, repeatability, recovery, detection limits and performance of negative and positive controls were assessed.
2 To apply the platform to different compartments of the water cycle, i.e. surface water samples from four abstraction points for the preparation of drinking water and effluent of a wastewater treatment plant (WWTP). The drivers of (anti-)androgenic, estrogenic and glucocorticoid activities in these samples were investigated.
3 To derive a workflow for the application of the platform in a context of legislative, governmental or routine monitoring.

2. Materials and methods

2.1. General procedure HT-EDA platform

A schematic representation of the experimental procedure of the EDA is provided in Fig. 1.

2.1.1. Extraction

Surface water samples and WWTP effluent were collected (for details, see section 2.3.1) and control samples were composed (see section 2.2.1). Samples were extracted using Oasis HLB solid phase extraction (SPE) cartridges in portions of 1 L (surface water and control samples) or 0.4 L (WWTP effluent) per cartridge. Compounds were eluted with 1:1 methanol (MeOH) and ethylacetate as described in (Houtman et al., 2018). For each sample, three extracts were prepared in this way. The three extracts were mixed and split again in three equal parts. Extracts (portions equivalent to 1 L for surface water or control samples; or to 0.4 L for WWTP effluent) were evaporated and dissolved in 50 µL dimethyl sulfoxide (DMSO, spectrophotometric grade, Acros, Geel, Belgium) for the analysis of the total activity in the unfractionated extracts (left part of Fig. 1). The second part was evaporated and dissolved in 700 µL milliQ water with 5 % MeOH for fractionation (in the centre of Fig. 1). The third part was stored at −20 °C for future use (shown at the right in Fig. 1).

2.1.2. Bioassay measurements in unfractionated extracts

Androgenic, estrogenic and glucocorticoid activities in unfractionated extracts were measured with the AR (agonistic), anti-AR (antagonistic mode), ERα and GR CALUX reporter gene assays (BioDetection Systems B.V., Amsterdam, the Netherlands) as described previously (Houtman et al., 2018; Van der Linden et al., 2008) and as laid down in the protocols of the supplier. We used the limits of quantification (LOQ) and limits of detection (LOD) for the analysis of unfractionated water extracts as derived and provided by BioDetections Systems. They are given in Table S1.The reference compounds were dihydrotestosterone (DHT), flutamide (Flt), 17β-estradiol (E2) and dexamethasone (Dex) respectively. A 10 point calibration curve of the reference compound was included on each plate. A sigmoidal standard curve (y = Bottom + (Top-Bottom)/(1 + 10^((LogEC50-x)*Hill Slope)) was fitted through the calibration curve using the software package GraphPad Prism 7.03 (www.graphpad.com) with y representing the luciferase activity, x the concentration of the reference compound and EC50 the half-maximum effective concentration. Responses of dilutions of the water extracts giving response between the limit of detection (LOD) and the EC50 were interpolated in the standard curve and responses expressed as equivalents of the reference compounds per litre water.

2.1.3. Fractionation

Chromatographic separation was performed by injecting 250 µL of the second extract part on a Waters Acquity UPLC C18 column according to (Zwart et al., 2020), with the following adaptations. The gradient changed from 5% MeOH in MilliQ-water at t = 0 min to 100 % MeOH in 23 min. Post column, the eluent was split into two portions. Two third was led to a FractioMate™ fraction collector (SPARKHolland & VU, Emmen & Amsterdam, the Netherlands,) (Jonker et al., 2019) and one third to an Impact II QTOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Fractions were collected at 4.8 s (288 fractions) intervals in transparent PS 384-well plates (Greiner Bio-One) to record the bioassay chromatograms (CALUX activities in the fractions). Prior to the fractionation, the 384-well plates were filled with 4 µL 10 % DMSO in milliQ water per well to serve as a keeper during solvent evaporation. The well plates containing the 288 collected fractions per sample extract were dried in a Centrivap concentrator for 5 h at 25 °C under vacuum until dryness and then stored at −20 °C.

2.1.4. Recording bioassay chromatograms with fractionated extracts

The cell suspensions (34 µL; ~10^5 cells/mL) were seeded in white 384 well plates with transparent colourless bottom (Greiner Bio-one, Frickenhausen, Germany) and incubated for 24 h at 37 °C and 5 % CO_2. The two outer rows were kept empty. The medium was removed and replaced with 24 µL assay medium. The assay medium used for plates seeded with AR CALUX cells for the anti-androgenicity testing was spiked with 3.4×10^{-7} M DHT in the well, to achieve a background level of DHT at approximately EC40 during exposure.

At the day of exposure, plates containing the fractions were thawed and fractions were redissolved in 50 µL assay medium containing 0.34 % DMSO. Each well containing a fraction was analysed with each reporter gene assay. Therefore, 10 µL of each fraction were added to the 24 µL medium in each well of the well plates of the different bioassays (AR, anti-AR, ER and GR CALUX). In this way, exposure was performed at a final DMSO concentration of 0.1 %. In order to save enough wells on each cell plate to include a calibration curve, the fractions were divided over two well plates per bioassay. Calibration curves (10 concentrations covering the full concentration response ranges) were included in triplicate on each well plate.

Well plates were exposed for 24 h and lysed with 17 µL lysis buffer (Houtman et al., 2004a) added to each well. The luminescence was measured with a luminometer (Tristar LB941, Berthold, Vilvoorde, Belgium). The luminescence was expressed as percentage of the maximum luminescence of the calibration curve and plotted as function of the retention time (RT) of each spotted fraction, resulting in the bioassay chromatogram.

2.1.5. MS analysis and identification

MS analysis and interpretation (non-target screening (NTS)) was performed on an Impact II QTOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) as described in (Zwart et al., 2020). Each sample was analysed twice; the first time in positive and the second time in negative ionisation mode. Data were recorded using data independent acquisition (broad band collision induced dissociation). MS data recorded within time windows between 0.2 min before and after peaks in the bioassay chromatograms were interpreted to identify compounds responsible for the observed activity. Extracted ion chromatograms (EIC) with accurate masses (± 5 m Da, full scan) were plotted for features. Criteria for assigning molecular formula and/or database entries to features were isotopic pattern matching expressed as mSigma score, and an absolute

mass accuracy window of 1 m Da, resulting in varying ppm accuracies across the mass range: 4 ppm at *m/z* 250, and 1 ppm at *m/z* 1000. RT and created fragments were derived from the EIC. The NORMAN Massbank High Resolution Mass spectral Database (https://massbank.eu/MassBank), and MzCloud Advanced Mass Spectral database (https://www.mzcloud.org) were searched for the features, to verify their identification according to the spectrum. If compounds were not available in these databases, fragments were uploaded in MetFrag (https://msbi.ipb-halle.de/MetFragBeta; 2 ppm) or Fragmentation Explorer in DataAnalysis (Bruker Daltonics, Billerica, MA, USA) to search for hits.

2.2. Evaluation of method performance

The performance of the EDA method was evaluated using positive and negative control samples, field samples and a spiking mixture. An overview of the test setup and the specific purposes of each step is given in Table S2.

2.2.1. Bioassay testing of unfractionated extracts

Positive (spiked clean water) and negative control samples (unspiked clean water, serving as procedure blank) were used to test the recovery of the activity from unfractionated extracts, and the repeatability of testing in triplicate

The positive controls were prepared by in triplicate spiking of 1 L water (HPLC quality, J.T. Baker, Fischer Scientific, Amsterdam, the Netherlands) with 50 µL of a spiking mixture in DMSO of the agonistic reference compounds of the ER CALUX (E2 1.8×10^{-8} M in the spiking mixture), AR CALUX (DHT, 1.60×10^{-7}M), and GR CALUX (Dex 1.3×10^{-5} M), that would lead to concentrations in water of 245 pg E2/L, 2.32 ng DHT/L and 245 ng Dex/L at 100 % recovery. Three separate portions of 1 L water were spiked with 50 µL of 2.84×10^{-3}M Flt in DMSO, leading to a concentration of 39.2 µg Flt/L water at 100 % recovery that was tested in the anti-AR CALUX. The antagonistic reference compound Flt was spiked separately from the agonistic reference compounds because spiked to the same bottles, DHT and Flt would mask each other's activities and recoveries would seem lower than they would be in reality.

Recovery of the activity was assessed by the measurement of the activity in the CALUX bioassays in the unfractionated extracts and expressing the measured activity as percentage of the activity at 100 % recovery. Coefficients of variation (CV) were calculated as: SD of a triplicate value / average of the triplicate value * 100 %.

The negative controls consisted of 3 × 1 L unspiked water of HPLC quality.

The repeatability of the CALUX analysis of a field sample was tested by in threefold sampling and processing of a surface water sample from the Lek Channel in the Netherlands The third replicate also served as Rhine water sample in the second goal of the study, see section 2.3.1).

2.2.2. Bioassay chromatograms (fractionated extracts)

Positive and negative samples were prepared in monoplo as described for unfractionated testing, with the difference that for the positive control now both agonistic and antagonistic reference compounds were spiked in the same sample, as they would be separated chromatographically during the fractionation. The control samples were analysed with the EDA platform as described in sections 2.1.3 to 2.1.5. The positive control served to test the recovery of the correct peaks in the bioassay chromatogram.

The negative control served to determine the background signal of the baseline and to calculate the minimum reporting limits (MRL) of the bioassay chromatograms.). The MRLs of the bioassay chromatograms for the 3 agonistic bioassays were calculated from the responses of all the fractions of the negative control sample as: average of the responses of the fractions of the negative control + 3x standard deviation (SD) of these responses. Due to the higher biological variation in the baseline (at ~EC40) of the anti-AR CALUX the same formula to calculate the MRL as for the agonistic assays would generate an unworkable MRL. Therefore the MRL for this assay was set as the average response minus 2x SD (approximately equalling 1/3 of the average response of the baseline).

In triplicate prepared extracts of 1 L portions of surface water from the Lek Channel were fractionated and bioassay chromatograms were recorded as described in 2.2.1, 2.1.3 and 2.1.4 to test the repeatability of the recording of bioassay chromatograms.

2.2.3. Consistency between bioassay chromatograms and MS results

Consistency between retention times of the peaks in the bioassay chromatogram and in the MS was investigated by in triplicate analysis of a spiking mixture containing 31 natural and synthetic androgens, estrogens, (gluco)corticoids and progestogens on the EDA platform (see Table S3). An aliquot of 12.5 µL of a 1 mg/L stock solution in MeOH was dissolved in 250 µL MQ and injected in triplicate on the UPLC-QToF-MS to determine RTs on the MS. Then, 12.5 µL portions of a 5 µg/L dilution of the stock solution were dissolved in 250 µL MQ and injected in triplicate (experiment a, b, and c) on the UPLC and fractionated to record bioassay chromatograms in AR, ER and GR CALUX.

2.3. Application to field water samples

2.3.1. Sampling and sample preparation

Surface water grab samples were collected in 1 L green glass bottles with PFTE-PE lined lids (Identipack, Someren, The Netherlands) prerinsed with ethyl acetate at abstraction points used by Dutch DW companies at the rivers Meuse (Enclosed Meuse) and Rhine (Lek Channel), Lake IJssel and a reclaimed land area Bethune Polder (Reclaimed Land). For a detailed description of the sites, see (Houtman et al., 2019). In addition, a 24 h volume proportional effluent sample was taken from a WWTP (for details, see description plant A in (9)). Samples were stored at 4 °C and processed with the EDA platform within one week. For surface water samples 3x a portion of 1 L water was extracted, for the effluent 3 × 0.4 L. In this way, one portion of extract of each sample served for bioassay analysis of the unfractionated extract, one for the EDA and one portion of extract was stored at −20 °C for future use.

In order to interpret the results of surface water and WWTP effluent in terms of risk assessment, the effects of the unfractionated extracts in the bioassays are compared to EBT values as proposed by Brand et al. (2013); Escher et al. (2018), 2015, Jarošová et al. (2014), Van der Oost et al. (van der Oost et al., 2017b) and Béen et al. (in preparation). Results of WWTP effluent were compared with ETBs for ecology, those of surface water with EBTs for ecology and, because of the function as drinking water source, with ETBs for human health as well.

2.3.2. Identity confirmation

Analytical standards of tentatively identified compounds were purchased in order to confirm their identity. A solution of 10 µg/L in 5% MeOH (injection volume 250 µL) of the standard was injected on the UPLC-QToF-MS to verify if the tentatively identified compound indeed eluted at the same RT as that of the peak in the bioassay chromatogram.

Series of 10 dilutions of identified candidate compounds were prepared in DMSO. These series were tested in triplicate in the anti-androgenic CALUX. The dilution series were also tested with the cytotox CALUX bioassay according to (van der Linden et al., 2014) to check if the anti-androgenic effect of the compounds was due to general cytotoxicity instead of genuine antagonistic action on the androgen receptor. If a compound was active, a sigmoidal curve (for the equation, see section 2.1.2) was fitted through the data to derive the EC50. The relative potency (REP) compared of the reference compound was calculated as:

$$REP_{(candidate\ compound)} = EC50_{(reference\ compound)}/EC50_{(candidate\ compound)}.$$

Table 1
(Anti-)androgenic, estrogenic and glucocorticoid activities (average +/− standard deviation) measured with CALUX bioassays 1.a.) negative (unspiked procedure blank) and positive (spiked) control samples and a surface water sample to determine recovery and repeatability in unfractionated extracts, 1.b. minimum reporting limits (MRL) of the peaks in bioassays chromatograms of the EDA-platform calculated from standard deviations in responses in the base lines and 2) activities in unfractionated extracts of field water samples. Activities in bold indicate attempts to identify responsible compounds with EDA.

CALUX	AR ng DHT eq./l	Anti-AR µg Flt eq./l	ERα pg E2- eq./l	GR ng Dex. eq./l
1.a. Method evaluation bioassay in unfractionated extract				
controls				
negative control (replicate 1, 2 and 3)	< 0.2	< 1.4	< 34	< 4.3
repeatability and recovery positive control				
replicate 1	1.8 ± 0.6	29 ± 3	238 ± 22	259 ± 61
replicate 2	1.5 ± 0.0	48 ± 12	161 ± 24	171 ± 5
replicate 3	2.3 ± 0.9	42 ± 4	169 ± 33	133 ± 5
average recovery pos. ctrl (%)	80 ± 18	101 ± 25	77 ± 17	73 ± 25
repeatability Lek Channel sample				
replicate 1	< 0.2	7.2 ± 1.0	< 34	< 4.3
replicate 2	< 0.2	6.1 ± 0.2	< 34	< 4.3
replicate 3	< 0.2	9.2 ± 0.9	< 34	< 4.3
average activity Lek Channel sample	< 0.2	7.5 ± 1.6 (CV 21 %)	< 34	< 4.3
1.b.Method evaluation bioassay in fractionated extract (bioassay chromatogram)				
MRL (% of max. induction by ref. compound)	5.8	17 (compared to ref.comp. DHT)	46	11
2. Field study				
samples (unfractioned extract)				
Enclosed Meuse	< 0.2	**72 ± 5**	~ 26 ± 2 < 34*	< 4.3
Reclaimed Land	< 0.2	10 ± 0	424 ± 17	< 4.3
Lek Channel	< 0.2	9.2 ± 0.9	< 34	< 4.3
Lake IJssel	< 0.2	7.4 ± 0.3	< 34	< 4.3
WWTP effluent	< 0.2	11 ± 1	376 ± 19	40 ± 5

* Value between LOD and LOQ.

3. Results and discussion

3.1. Evaluation of the method performance

3.1.1. CALUX analysis in unfractionated extracts

The (anti-)androgenic, estrogenic and glucocorticoid activities measured in the unfractionated control samples are presented in Table 1. The activities in the negative control samples were below the limit of detection, indicating that no contamination with active substances had occurred during the experimental procedures. The recoveries of the agonistic and antagonistic activities in the positive controls were all > 70 % of the spiked concentrations, indicating that the used extraction procedure recovers steroid hormone-like and antagonistic compounds, and with repeatabilities between 17 % (ERα) and 25 % (anti-AR and GR).

The repeatability of anti-androgenic activity in the triplicate water samples from the Lek Channel (CV 21 %) was comparable with that of the positive control (CV 25 %, that contained much less matrix). The samples showed no androgenic, estrogenic or glucocorticoid activity, therefore the repeatability for these effects could not be judged.

3.1.2. Bioassay chromatograms

The bioassay chromatograms of the negative and positive controls can be found in the SI (Fig. S2). As expected, there were no peaks in the bioassay chromatograms of the negative control. All reference compounds were detected in the positive controls as single sharp peaks in the bioassay chromatograms. DHT and Flt could be detected as individual peaks and there was no mutual masking of their activities, as would have been the case in an unfractionated extract in which they would have been present together. This indicates the added value of fractionation if both agonistic and antagonistic compounds for a certain bioassay are present in a sample. The triplicate bioassay chromatograms of the anti-androgenic activity in the Lek Channel sample are presented in Fig. S3. Due to the addition of DHT to the exposure medium of the anti-AR CALUX, the baseline of the bioassay chromatograms of this assay was higher and showed higher biological variation than seen in the AR, ER and GR CALUX assays with baselines at EC0. Nevertheless, the repeatability of the anti-AR bioassay chromatogram was satisfactory: a negative peak of anti-androgenic activity was clearly seen in all triplicates at a retention time of 16.1 ± 0.2 min (CV1.4 %).

The MRLs calculated for peaks in bioassay chromatograms are given in Table 1, part 1b. As there were considerable differences between assays in the experimental variation of the baseline response, the MRLs differ per assay.

3.1.3. Consistency between bioassay chromatograms and MS results

The repeatability of the fractionation was also assessed chemically by injecting a spiking mixture of 31 steroid hormones in triplicate on the UPLC-MS system. The compounds eluted between 9.2 and 17.0 min and maximum differences between RT triplicate values were 0.5 % (0.085 min). The acquired RTs and the CVs are given in Table S3, together with literature values for relative potencies of the compounds in the AR, ER and GR CALUX assays. The bioassay chromatograms that were recorded in triplicate in parallel for this mixture with AR, ER and GR CALUX are shown in Fig. 2. Most compounds in the mixture were detected in peaks of the bioassay chromatograms. Progestogens that lack sufficient cross reactivity on AR, ER and GR receptors were not visible in these assays. In addition, cortisone, prednisone and aldosterone had too little potency on the GR receptor to be detected in the GR CALUX assay at the tested concentrations (Relative potency < 0.008 compared to dexamethasone; Table S3). Due to the fact that some compounds had RTs close to each other and that peak widths of single eluting compounds on this system were often > 0.3 min, co-elution of was observed e.g. for many glucocorticoids in the GR CALUX bioassay chromatogram and for 17α- and 17β-E2, estrone (E1) and ethynylestradiol (EE2) in the ER CALUX bioassay chromatogram. As can be seen in Fig. 2, a very good overlap was observed between the triplicate chromatograms with regard to peak RTs (CV ≤ 1.8 %, see

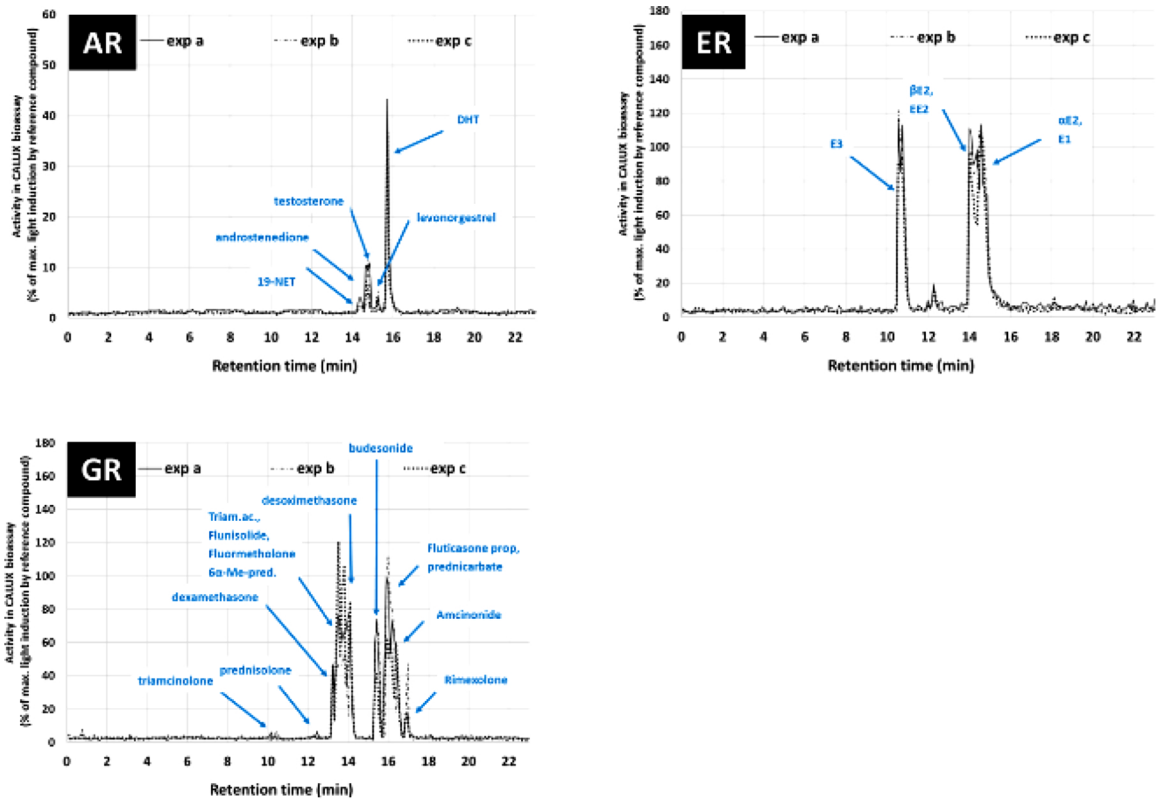

Fig. 2. AR, ER and GR bioassay chromatograms of fractionated (n = 3), spotted and CALUX analysed mixture of steroid hormones. Small peaks related to spiked compounds indicated in Table 1 are annotated even if they were detected below the respective MRL.

Table S4) and peak surface (total average peak surfaces were AR: 8.5 ± 1.8; ER: 129.2 ± 10.7 and GR: 119.4 ± 10.5 min*activity).

The high repeatability of the fractionation and consistency between the peaks in the bioassay chromatograms and detected by MS indicate that the bioassay chromatograms can indeed be used to direct the identification process in EDA, and that only the MS data within small RT window around bioassay peaks have to be interpreted in the search for the causative compounds.

3.2. Application of EDA platform to water samples

3.2.1. Unfractionated extracts

The total (anti)-androgenic, estrogenic and glucocorticoid activities measured in unfractionated extracts of the water samples are shown in Table 1, part 2. All activity types, except androgenic, were observed in the WWTP effluent. This is in line with earlier work in which removal of androgenic activity to undetectable levels in effluent of the same WWTP was observed whereas anti-AR, ER and GR were still detected in effluent (Houtman et al., 2018). Also other studies investigating municipal WWTPs reported reduction or removal of androgenic activity in the treatment, and the presence of anti-androgenic and estrogenic activity in effluent (Bain et al., 2014; Itzel et al., 2020).

The anti-androgenic activity (11 ± 0) ng Flt eq/L) was below the EBTs for ecological risk of Van der Oost et al. (van der Oost et al., 2017b) and Escher et al. (2018) (25 and 14.4 µg Flt eq/L respectively). The estrogenic activity in effluent (376 ± 19 pg E2 eq/L) was within the range of the EBTs published for the ecological risks of this type of activity (between 0.1 and 0.4 ng E2-eq/L) Escher et al. (2018); van der Oost et al., 2017b; Jarošová et al., 2014). This indicates that – without further dilution in receiving waters- this effluent might lead to estrogenic effects in the ecosystem. The glucocorticoid activity (40 ± 5 ng Dex eq/L) was below the only available EBT for the ecological risk of glucocorticoid activity (100 ng Dex eq/L (van der Oost et al., 2017b).

Estrogenic and anti-androgenic activities were detected in surface waters at abstraction points for DW production, whereas AR and GR activities were not. A higher estrogenic activity than at the other locations was found at the Reclaimed Land. Like in the WWTP effluent, the estrogenic activity in the Reclaimed Land water sample was in the range of the ecological EBTs. Concerning human health risk, the activity was below the EBT published by (Brand et al. (2013)) (3.8 ng E2 eq/L), but higher than the values published by (Escher et al. (2015)) and proposed by Béen et al. (0.25 ng E2 eq/L; in preparation). At other surface water locations, estrogenic activity was below available EBTs. The highest anti-androgenic activity was found at the Enclosed Meuse. At this location, the anti-androgenic activity exceeded considerably the EBTs both for ecological (Escher et al., 2018; van der Oost et al., 2017b) and human health risks (Escher et al., 2018) and the value proposed by Béen et al. (2020, in preparation) of 4.8 µg Flt-eq/L. At other surface water locations, anti-androgenic activity was below available EBTs.

In summary, ecological risks of anti-androgenic and estrogenic activities cannot be excluded at some of the investigated locations. These activities exceed (some of the) EBTs for human health as well. However, it should be noticed that the drinking water companies using these locations as their sources apply advanced multi-barrier treatment technologies to remove organic pollutants - including those with endocrine disrupting potencies - to undetectably low levels or levels with negligible risks (Houtman, 2010).

3.2.2. ER bioassay chromatograms

Bioassay chromatograms were recorded for the water samples with the highest activities (WWTP, Enclosed Meuse and Reclaimed Land). Bioassay chromatograms for the WWTP effluent are presented in Fig. 3, those for the Enclosed Meuse and Reclaimed Land in Fig. S4 of the SI.

A large peak of estrogenic activity was found between 13.9 and 15.4 min in the WWTP bioassay chromatogram. Analysis of the MS-data did not lead to identification of the causative compound(s). However,

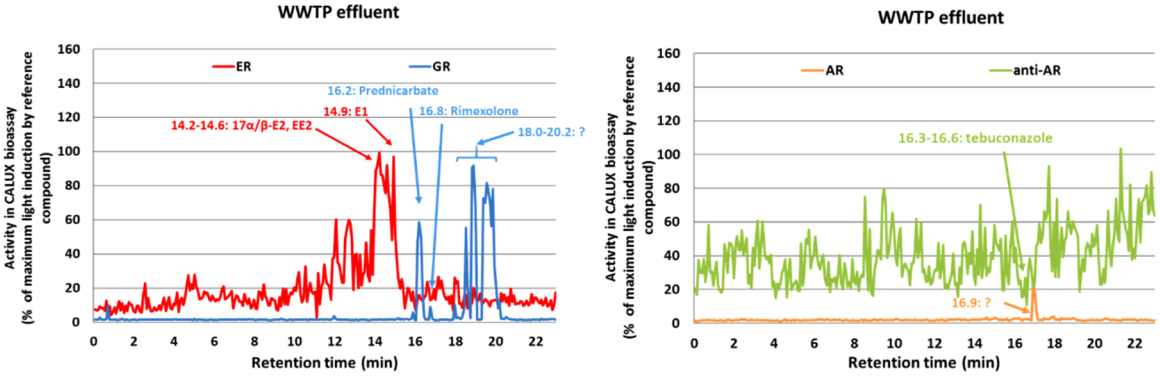

Fig. 3. bioassay chromatograms of the WWTP effluent sample for estrogenic and glucocorticoid activity left) and (anti)-androgenic activity (right). Tentatively identified compounds are annotated at the corresponding RTs (min). The hormones were identified according to the corresponding RT, tebuconazole was tentatively identified based on RT and mass spectrum similarity.

from many studies it is known that the natural estrogens 17α- and 17β-E2, E1 and the synthetic estrogen and contraceptive EE2 are widespread in the aquatic environment. They are excreted by humans and cattle and as such predominantly emitted by WWTPs (Johnson et al., 2000; Petrovic et al., 2002) and farm land run-off (Johnson et al., 2006). They appear to be responsible for the estrogenic activity at many locations and in many matrices (Purdom et al., 1994; Houtman et al., 2006, 2007; Houtman et al., 2004b). As the retention times of these estrogens are RT = 14.20 min (17β-E2), 14.40 (EE2), 14.57 (17α-E2), and 14.90 min (E1; Table S3), it is very likely that the large estrogenic peak observed in the bioassay chromatogram of the WWTP effluent is a co-elution of the mentioned estrogenic steroids. The estrogenic bioassay chromatograms of the Enclosed Meuse and Reclaimed Land (Fig. S4) also show peaks around RT = 14 min, endorsing the tentative identification of these compounds. The fact that the estrogens were not identified by the high resolution MS can be explained by the much higher sensitivity for steroid estrogens in the ERα CALUX bioassay. The very low concentration (0.4 ng EEQ/L in the unfractionated extract) already evokes a clear response in the bioassay but is too low to be visible in the NTS mode of the HRMS used.

3.2.3. GR bioassay chromatograms

Six distinctive peaks were found in the bioassay chromatogram of the glucocorticoid activity. The high resolution of the fractionation in our HT-EDA platform thus indicates that at least six different compounds contributed to the glucocorticoid activity. Similarity in RTs with compounds from the standard mixture was observed for prednicarbate (RT = 16.2 min) and rimexolone (RT = 17.0 min, Table S3). These are pharmaceuticals prescribed, respectively, to treat skin and eye inflammations. The presence of glucocorticoids in WWTP effluents has been reported earlier (Houtman et al., 2018; Zwart et al., 2020; Schriks et al., 2010). And using EDA, it often appeared difficult to identify all responsible compounds (Zwart et al., 2020; Hashmi et al., 2020).

Probably due to the same sensitivity issue as for estrogens, the MS failed to tentatively identify candidate compounds for the other four peaks. In this study, unidentified peaks were detected in the last part of the bioassay chromatogram (RT 18.0–20.2 min). As mentioned above, it is often observed that glucocorticoid activity is not fully removed during (conventional) wastewater treatment. These peaks might be caused by glucocorticoids that are either not degraded or absorbed in the WWTP, or they might consist of active metabolites of glucocorticoids formed during treatment (Zwart et al., 2020). In the future, we will investigate this by comparing bioassay chromatograms of samples of the different steps of the WWTP. The bioassay chromatograms of the surface water sites (Fig. S4) did not show any peaks of glucocorticoid activity. This is in line with the absence of glucocorticoid activity in the corresponding unfractionated extracts.

3.2.4. Anti-AR bioassay chromatograms

The anti-androgenic bioassay chromatogram of the WWTP sample showed a negative peak at 16.3–16.6 min. The androgenic bioassay chromatogram showed a small androgenic peak (that was not further identified) at 16.9 min. This latter activity must have been masked by the anti-androgenic activity in the unfractionated extract. Fractionation thus helped separating agonistic and antagonistic compounds, which are not observed in whole samples due to mutually counteracting activities (Weiss et al., 2009). Analysis of the MS data in the time window 16.3–16.6 min revealed a compound with an accurate mass of 307.1451 amu; this mass and six most abundant fragments were uploaded in MetFrag, leading to tebuconazole as single hit (KEGG database). Consequently, we tested this azole fungicide as pure standard, and the RT was confirmed on the MS to be 16.3 min, and as such the RT matched with that of the bioassay chromatogram peak. A concentration series of tebuconazole was tested in the anti-AR and cytotox CALUX bioassays and the concentration-response curves are shown in Fig. 4.

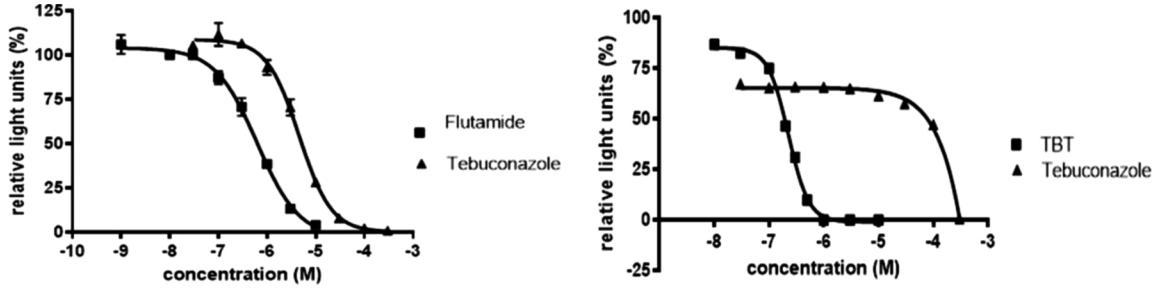

Fig. 4. Concentration-response series of tebuconazole in the anti-AR CALUX bioassay (left) and the cytotox (right) CALUX bioassays. Tebuconazole shows anti-androgenic activity and at a ~100 times higher concentration also cytotoxicity.

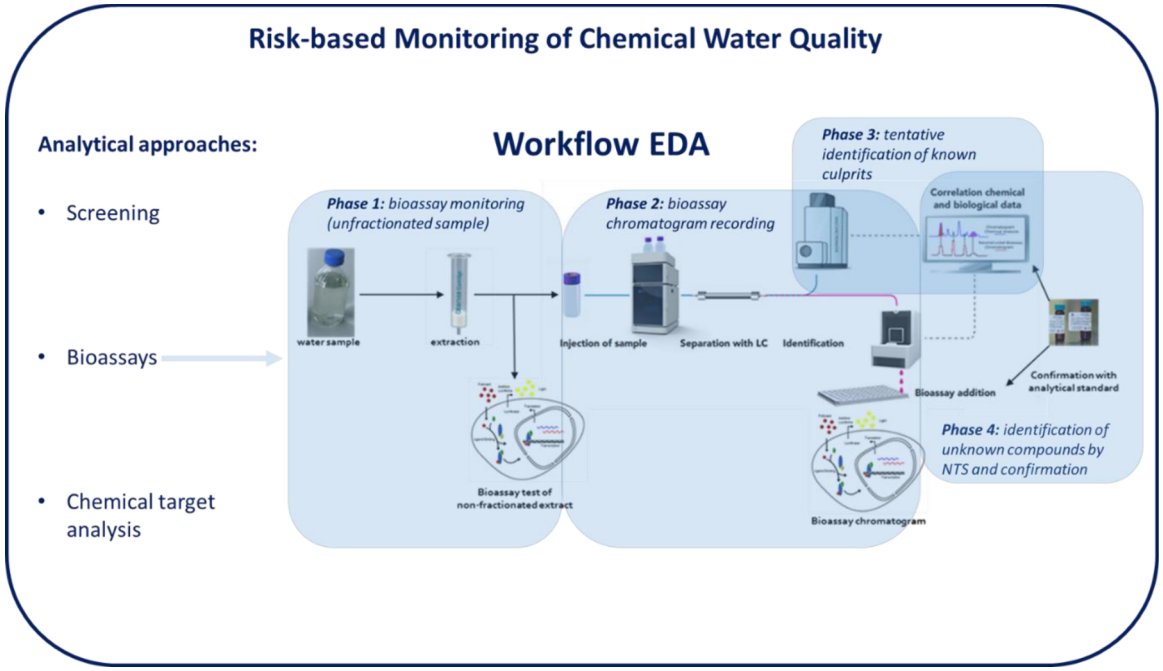

Fig. 5. Schematic representation of the position of analytical approaches in risk based monitoring of chemical water quality and the workflow of EDA as proposed for a routine monitoring context. In this workflow, EDA is split up in phases (blue boxes). Depending on the research question and the results obtained, it can be decided if only phase 1 (bioassay monitoring in unfractionated samples) or more phases of the platform, eventually aiming at full identification of active compounds, need to be performed.

Tebuconazole proved to be anti-androgenic with an EC50 of 4.4 ± 0.1 μM and a REP of 0.13 ± 0.01 compared to that of Flt (left panel). This is in line with findings of Seeger et al. (2016) who found and EC10 of 0.9 μM for tebuconazole in the anti-AR CALUX and of Christen et al. (2014) who found an EC50 of 6.86 μM in the human mammary gland cancer cell line MDA-kb2. Tebuconazole appeared to be cytotoxic at concentrations $> 10^{-4}$ M (Fig. 4, right panel). This means that its anti-androgenic activity is apparent at much lower concentrations (~100-fold) than its cytotoxicity, indicating that the inhibition of signal by the sample extract in the anti-AR CALUX is due to real antagonism on the AR, and as such that tebuconazole is confirmed as anti-androgen in the WWTP effluent extract. Many environmental contaminants, including polychlorinated biphenyls (Hamers et al., 2011), pesticides (Aït-Aïssa et al., 2010) and pharmaceuticals (e.g. Flt), are known to have anti-androgenic potencies. Propiconazole - an azole fungicide just like the now identified tebuconazole – was identified in surface water in former demonstration studies with the EDA platform (Zwart et al., 2018).

The anti-AR bioassay chromatogram of the Enclosed Meuse (Fig. S4) showed a negative peak at 16.1 min, which was in line with the anti-androgenic activity in the unfractionated extract (Table 1). Analysis of MS data at this RT identified fatty acids and sulfonic acids that are unlikely to interfere with the AR receptor but also capsaïcin. This component of chilli pepper was found to induce degradation and inactivation of the AR in prostate cancer cells (Zheng et al., 2015). However, a dilution series of the pure compound did not show anti-androgenic activity in the anti-AR CALUX at concentrations up to $3*10^{-4}$ M (Fig. S5). Therefore, the compound does not explain the anti-androgenic activity in the Enclosed Meuse, and the real driver of the anti-androgenic activity at this location remains to be elucidated.

The anti-androgenic bioassay chromatogram of the Reclaimed Land did not show a clear negative peak, indicating that the anti-androgenic activity in the unfractionated extract of this sample might e.g. have been due to multiple compounds with low individual activities that, also considering the high biological variation in the baseline of the bioassay chromatogram, did not evoke significant negative peaks.

3.3. Workflow for the application of the platform in a routine monitoring context

Water boards, DW companies, and governments put large efforts in monitoring chemical water quality. For a long time, chemical water quality monitoring has relied on *chemical target analysis* alone. This is mainly due to the fact that water quality legislation often requires target compound analysis methods for chemical monitoring as well as due to the analytical state of the art. However, there are too many contaminants in the aquatic environment to be monitored individually. Moreover, many possibly relevant compounds are unknown and as such cannot be incorporated in target methods (Brack et al., 2016). The really important question in risk assessment is if the water is safe for humans and the ecosystem, and not whether we can create a virtually complete list of all present compounds with their concentrations. Fortunately, a paradigm shift from 'compound' based to 'risk' based monitoring is currently ongoing. The 2015 update of the European Drinking water Council Directive 98/83/EC Annex II grants a certain degree of flexibility in performing the regular monitoring. Member States are allowed to derogate from their monitoring programs, provided credible risk assessments are performed. This opens the door for the implementation of *bioassays* and *screening* techniques to focus on the most relevant drivers of chemical risk at each water body functioning as DW source. Concerning the Water Frame Work Directive, the target analysis of priority compounds is still compulsory, however, the selection of sites with the highest contaminant risks can be done by alternative methods, e.g. using an effect-based approach using bioassays and EBTs (Escher et al., 2018; Brack et al., 2017) as for example in the smart integrated monitoring (SIMONI) strategy for environmental risk assessment (van der Oost et al., 2017b).

Hence, bioassays and screening will play a more prominent role in (the design of) future risk-based monitoring programs (Brack et al., 2016; Houtman et al., 2011), with EDA as the ultimate combination to focus on and prioritise compounds driving risks. Chemical target analysis will still be applied for the dedicated and quantitative monitoring of specific groups of compounds of particular interest, but will play a

less prominent role. The position of analytical approaches in a risk-based monitoring program is shown schematically in the "Analytical approaches" in Fig. 5.

The at-line fractionation with a spotting device as incorporated in our EDA platform has made EDA much less time-consuming than the 'classic' manual EDA approaches. However, performing a full EDA study is still time and cost intensive. We therefore propose a workflow by which EDA is split into phases and the information created by each phase is exploited to the maximum. In this way, depending on the research question, it may not always be necessary to proceed through all steps of the platform. The workflow is represented in the "Workflow EDA" in Fig. 5, and is elaborated in sections 3.3.1 to 3.3.4.

3.3.1. Phase 1: bioassay monitoring

Bioassays are more and more used to monitor water quality for compounds with biological activities. In a routine monitoring context, initially only the bioassay measurements in the unfractionated extract will suffice. If measured bioassay responses exceed an EBT and the causative compounds are unknown, continuation with phase 2 is needed for both human and environmental risk assessment. It can also be decided to continue with phase 2 if the activity is $<$ EBT and specific questions arise that may be answered by bioassay chromatograms (see 3.3.2). One such question might arise if there are both agonistic and antagonistic compounds in the sample that mutually mask each other's activities to a combined level $<$ EBT.

The division of EDA phases is already incorporated in the SIMONI strategy for environmental risk assessment (van der Oost et al., 2017b). Tier 1 of this strategy assumes that the risks are low if no EBT exceedances are observed in a battery of relevant *in vivo* and *in vitro* bioassays. In that case no additional research is required. In cases where EBT exceedances indicate increased environmental risks, SIMONI applies target analysis of specific groups of organic substances (tier 2) and phases 2–4 of the EDA workflow to elucidate the drivers.

3.3.2. Phase 2: bioassay chromatogram determination

The second phase of the EDA workflow is the fractionation and recording of bioassay chromatograms. Simultaneously, MS data are recorded, although they do not have to be analysed or interpreted yet in this phase. They can be archived and used for (retrospective) analysis in later phases of the workflow. The bioassay chromatogram itself already provides valuable information to characterize the drivers of the observed effect. Due to the high resolution of the fractionation, compounds elute over multiple fractions, generating a peak of several data points for each compound, although co-elution may lead to some overlap. This means that the number of peaks represents the (minimum) number of compounds contributing to the observed activity. In addition, if both agonistic and antagonistic modes are recorded, the bioassay chromatograms show the presence of agonistic compounds whose effects are masked by antagonistic compounds in the non-fractionated extracts (and vice versa). Moreover, the RTs of the peaks indicate the polarity of the compounds (or any other principle the chromatographic separation was based upon).

By plotting overlays, bioassay chromatograms can be used to mutually compare samples taken at different sites. By recording bioassay chromatograms at a single site over time, variations in water composition regarding a specific activity can be investigated. By recording chromatograms after several water treatment steps, fate of the effects in treatment plants can be followed. If it is necessary to identify responsible compounds, continuation to the next phase(s) is needed.

3.3.3. Phase 3: tentative identification of known culprits

The most time-consuming phase of the platform is the NTS identification (phase 4). However, as was demonstrated in the field study for estrogens and certain glucocorticoids, some suspects may already be found in the bioassay chromatogram. They can be tentatively identified by their known RTs and added to a suspect list. Sometimes this is the only possible way of identification, because for certain compounds (such as highly potent steroids) the NTS is not sensitive enough to detect the low amounts that cause activity in the bioassays. In those cases that the drivers of the effect are fully unknown, phase 4 is entered.

3.3.4. Phase 4: HRMS identification of unknown compounds by NTS and confirmation

NTS is gaining strength to identify compounds (Hollender et al., 2017). A large advantage of application of NTS in the context of EDA is that the interpretation, the most time-intensive part of NTS, exclusively has to be done within the RT-window in which the peak of interest eluted. As seen in section 3.1, we obtained an excellent consistency between the RTs of peaks in the bioassays chromatogram and MS data. Only features within a small window of $0.1-0.2$ min around the RT of the bioassay peak will have to be interpreted. NTS in the context of EDA, however, is no guarantee for success: in our study tebuconazole was successfully identified in the WWTP effluent, but the anti-androgenic activity in the Meuse sample could not be addressed. Generally, identification will be especially challenging for the more 'promiscuous' effects, such as anti-androgenicity and e.g. pregnane X receptor activation, for which a wide variety of compounds in the environment may be responsible. For such endpoints the chance is small that specific culprits can be denoted beforehand, so NTS is essential. The NTS technique is still developing rapidly, so its potency will expectedly increase further in the near future.

4. Conclusion

The developed high resolution, high throughput EDA platform used in this study enables characterisation and identification of biologically active compounds in water samples. The performance evaluation showed that the EDA platform is robust, delivers very repeatable results and excellent consistency between the bioassay chromatograms and MS results.

The application of the platform to field samples led to the tentative identification of estrogens, anti-androgens and glucocorticoids in wastewater treatment plant effluent and surface water samples, although not all bioassay peaks could be assigned to their causative compounds.

The field study demonstrated that a full identification and confirmation of the drivers of the biological activity is not needed in all cases to characterize the active compounds present. Even without the MS results the bioassay chromatograms give extensive added information, e.g. they inform us about the active compounds in terms of polarity (low or high RT), number and activity of contributing compounds (number of peaks and peak area). These aspects are used in the workflow that is proposed for the application of the EDA platform in a routine monitoring context. As such, the EDA platform is a valuable tool for the investigation of unknown compounds with biological activity, both in an academic setting as in the context of legislative, governmental or routine monitoring.

CRediT authorship contribution statement

Corine J. Houtman: Conceptualization, Methodology, Formal analysis, Resources, Writing -original draft, Visualization, Supervision, Project administration, Funding acquisition. **R. ten Broek:** Methodology, Software, Validation, Investigation. **Y. van Oorschot:** Validation, Investigation, Visualization. **D. Kloes:** Validation, Investigation. **R. van der Oost:** Writing - review & editing. **M. Rosielle:** Validation, Investigation. **M.H. Lamoree:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Acknowledgements

This study was performed by assignment of the Dutch drinking water companies Dunea, PWN and Waternet. Waternet is acknowledged for the possibility to sample at one of its WWTPs. The authors are indebted to Nick Zwart, Eelco Pieke and Bram Veefkind for their valuable contributions. BioDetection systems B.V. is acknowledged for providing the AR, ER, GR and cytotox CALUX cell lines.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.etap.2020.103460.

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